Annex 2
Test protocol

Royal Netherlands Institute for Sea Research

TEST PROTOCOL AND QUALITY ASSURANCE PROJECT PLAN (QAPP) FOR THE BIOLOGICAL EFFICACY TESTING OF THE AQUATRICOM BALLAST WATER TREATMENT SYSTEM (AQUAWORX, GmbH) AS PART OF THE TYPE APPROVAL PROCESS UNDER RESOLUTION MEPC 125.53 AND MEPC 174.58

CONFIDENTIAL

until further notice

Submitted to the

Federal Maritime and Hydrographic Agency (BSH)

January 2010

Note: The outline of the test protocol is based on the requirements according to the IMO guidelines G8. However, as the reader may notice testing will much be broader and more in depth. We have also planned additional experiments, like incubation experiments under favourable growth conditions and testing the efficacy of the treatment for organisms which are officially also not part of the G8 guidelines. More experiments may be included, although not strictly necessary in the current version of G8, if we think this is relevant for the efficacy or environmental effect studies. The reason for this D2+ (NIOZ-D2+ standard) testing is new legislation, e.g. state of California, which is more strict than the present IMO D2-Standard.

1 INTRODUCTION

The test protocol detailed below should form the basis for the biological efficacy testing of the AquaTriComb™ Ballast Water Treatment System (BWTS), produced by Aquaworx ATC GmbH. The tests outlined in this protocol will evaluate the biological efficacy of the AquaTriComb™ BWTS as outlined in the Guidelines for Approval of Ballast Water Management Systems, G8, adopted on 22 July 2005 as Resolution MEPC.125/53 (Anonymous 2005) and amendments MEPC 58 (Anonymous 2008).

The test protocol will be submitted by the applicant (Aquaworx ATC GmbH) to the German National Administration for approval (Federal Maritime and Hydrographic Agency BSH). The land-based tests will be conducted according to the test outlined in this document.

The AquaTriComb™ Ballast Water Treatment System (BWTS) applies for Type Approval through German authority and therefore conducting land based tests according G8 of the Ballast Water Convention of the IMO.

The Basic Approval was given to the AquaTriComb™ system during IMO/MEPC 59 meeting, July 2009 in London. In December 2009 the national authority BSH decided on the base of comprehensive ecotoxicological tests and chemical water analysis, that during the treatment process of AquaTriComb™ no chemicals are added or produced. On this basis Aquaworx got the permission from BSH to follow G8 for the Type Approval process. Following the requirements of BSH the dossier of the type approval will follow the formalities and written form of a Final Approval and will we submitted to MEPC as a information paper.

Aquaworx ATC GmbH works very close with the German class society Germanischer Lloyd (GL, Hamburg) regarding the construction of the BWT system to achieve a statement from the GL as part of the approval process.

The AquaTriCombTM BWTS is composed of pre- and after-treatment modules to disinfect aquatic species in the ballast water of ships. The treatment system comprises mechanical filtration with a self-cleaning suction process as the pre-treatment step. The after-treatment compose ultraviolet-C (UV-C) radiation in combination with ultrasound for self-cleaning of UV-C Sleeves.

Due to the highly efficient combination of US oscillation and UVC radiation the AquaTriComb™ technology can abstain from any other water conditioning or chemical additives, which may pose a hazard to humans and / or to the environment. Because the US and UVC technology is precisely adjusted to avoid the generation of OH• radicals, the concentration of the OH• radical related by- or end products is that low that risks to human health and to the environment can be demonstrably excluded.

2 GENERAL ASPECTS

2.1 Profile *of Royal*-NIOZ

All tests of the AquaTriComb™ BWT-System are going to be carried out under the supervision of the Royal Netherlands Institute for Sea Research, Landsdiep 4, 1797 SZ 't Horntje, Texel, the Netherlands (from hereon NIOZ).

Short description of NIOZ (for details see: www.nioz.nl)

NIOZ Royal Netherlands Institute for Sea Research is the National Oceanographic Institute of the Netherlands. NIOZ is part of the Netherlands Organization for Scientific Research (NWO). The institute employs around 200 people and the annual budget is approximately €20 million.
The mission of NIOZ is to gain and communicate scientific knowledge on seas and oceans for the understanding and sustainability of our planet. The institute also facilitates and supports marine research and education in the Netherlands and in Europe.

In order to fulfil its mission, the institute performs tasks in three specific fields.
Research: The emphasis is on innovative and independent fundamental research in continental seas and open oceans. The institute also carries out research based on societal questions when this merges well with its fundamental work. The senior scientists at NIOZ all participate in international research projects.
Education: The institute educates PhD and other students of universities and schools for professional education. Together with universities NIOZ also organises courses for PhD students and master students in the marine sciences. A number of our senior scientists of NIOZ are also appointed as professors at the Dutch and foreign universities.
Facilitary services: NIOZ invites marine scientists from Dutch and foreign institutes and universities to write scientific proposals involving the institute's research vessels, laboratories, and the large research equipment, which is often designed and built by the institute's own technical department.
The basic oceanographic **disciplines** studied at NIOZ are physics, chemistry, biology and geology. Multidisciplinary research is regarded as one of the main strengths of NIOZ.

NIOZ has extensive experiences in the field of ballast water and ballast water treatment technologies. During the past 5 year several pilot test for ballast water treatment we conducted in the NIOZ harbour and in 2007 a full scale land-based testing was carried out for Final and Type Approval. For details read Veldhuis et.al. (2006) and **www.nioz.nl**.

Figure 1: aerial view of NIOZ harbour (lower right), NIOZ (upper left) and TESO ferry (top).

2.2 Profile of Aquaworx ATC GmbH

Aquaworx ATC GmbH, www.Aquaworx.de, founded in 2001, has developed proprietary ballast water treatment systems that have been specifically designed to safely and economically eliminate the worldwide transfer of aquatic invasive species.

- The Aquaworx ATC GmbH has its headquarters in Munich, Germany and was founded in October 2008. Mr. Peter Falk, an experienced industry and sales professional, is leading the Aquaworx ATC GmbH as managing director. Aquaworx markets its solutions worldwide from Germany and has currently five employees in Switzerland and ten in Munich. The technical development of diverse Aquaworx products began in 2005. The first big success was a very promising trial of a wastewater processing plant in Altenrhein in Switzerland. This plant was already working according to the principle of combining UV light with ultrasound. In April 2008 the decision was made to step into the ballast water treatment field. The first ballast water treatment test plant was designed and built in Switzerland and tests have been conducted successful in biological and technical aspects since September 2008 at the Lloyd Werft Bremerhaven.

Aquaworx ATC GmbH is in collaboration with the Royal Netherlands Institute for Sea Research (NIOZ) to conduct certification testing also together with the German Federal Maritime Agency (BSH), who will act as the administrating agency during the certification and Type Approval process.

Technical Overview of AquaTriComb^{TM}

Aquaworx ATC GmbH has developed the ballast water treatment system AquaTriCombTM comprising pre- and after treatment modules. The capacity of the volume flow rate can be designed by means of aligned up-scaling of the modules.

The AquaTriCombTM BWTS comprises mechanical filtration with a self-cleaning suction process as the pre-treatment step. The after-treatment compose ultraviolet-C (UV-C) radiation in combination with ultrasound for self-cleaning of UV-C Sleeves.

Due to the highly efficient combination of US oscillation for cleaning and UV radiation for disinfection the AquaTriCombTM technology can refrain from any preparations or chemical additives, which may pose a hazard to the population and / or to the environment.

Because the US and UV-C technology is precisely adjusted to avoid the generation of OH• radicals, the concentration of the OH• radical related Other Chemicals (acc. to G9) is that low that risks to human health and to the environment can be demonstrably excluded.

During ballast water intake, the pre-treatment stage for removal of particles and organisms, the ballast water is filtered through one MicroSintFilter (MSF) module. All elements of the filter cleaning technology are developed by Aquaworx. The filtration technology with 20 µm pore size allows very effective removal of particles and organisms. The automatically self-cleaning filter process is based on permanent suction technology by creating under pressure on the filter surface. During filtration one filter is permanently producing treated ballast water. During the production stage of this filter, the other identical filter is in stand-by mode. Switching over from one filter to the other is controlled by differential pressure of the filter in production. In case the filter efficiency of the main filter decreases due to the increased pressure drop over the filter, the stand-by filter takes over the filtration process. The main filter will be cleaned during it is in stand-by mode.

After the filtration stage, the water is exposed to monochromatic UV light inside the UV-reactors. The low pressure mercury germicidal lamps produce UV-C light at predominantly 254 nm. Cleaning of the lamp protecting quartz sleeves is done periodically by using low frequency ultrasound. The application of low pressure UV-technology at 254 nm wave length disinfects aquatic organisms very effectively without generating any chemicals.

The most important impact of the ultrasound is the highly effective cleaning of the UV-Quartz sleeves through the ultrasonic transmission along the full length of the UV-reactor, avoiding accumulations of bio films and/or inorganic salts on the quartz sleeves, assuring that the produced UV light is able to penetrate the water to achieve the required killing rate of the organisms. During the disinfection treatment inside the UV-reactor the organisms are lethally affected by UV-light.

During de-ballast operations the ballast water from the tanks is again treated by the complete pre- and after treatment plant before discharge. Organisms, e.g. bacteria and mono-cellular phytoplankton, which in spite of effective treatment may grow again during long ship voyages, will be effectively killed. Backwash water is treated during a special developed process. During filtration of de-ballast water the backwash valves are closed, therefore backwash is not discharged over board. After an intermediate storage in a transfer container, the backwash is treated by the first UV reactor. Because of the low flow rate of approx. 15-20 m^3/h the residence time of the backwash water in the UV reactor is high, therefore it is treated with a high UV dose. This results in an extremely effective treatment through the high energetic impact of UV radiation. After the special treatment of the backwash water it is added to the main filtration stream.

Sampling points are designed in between the ballast water pump and the treatment plant, in between the pre- and after treatment, and after the disinfection step. During de-ballast operations an additional sampling point after the first reactor is installed to analyze the quality of the treated backwash water.

The piping diameter of the sampling points will be 1/8 of the ballast water pipe diameter, i.e. 250/8 = 31,25 mm. All valves are fitted with flanges, and electrical-driven and manual actuators.

In case of repair and maintenance or malfunction the complete treatment plant can be shut-off with safety valves and / or by-passed with by-pass valves.

Specific features/advantages of the AquaTriComb™ ballast water treatment systems

The advantages of the AquaTriComb™ ballast water treatment plant are summarized as follows:

- Radical abandonment of any Preparations and avoidance of reactive intermediates
- Precisely adjusted US and UV-C technology to avoid the generation of OH• radicals and therefore OH• radical related Other Chemicals
- The application of low pressure radiation (254 nm) and low frequency ultrasound with extremely short termed ultrasound impact time (< 1 s), based on analysis of treated water, none Other Chemicals during the treatment process are generated
- Pore size of sinter filter (MSF modules): 20 µm
- During de-ballast operations the water is filtered and disinfected by means of pre-treatment (filter) and after-treatment (ultraviolet radiation)
- The application of ultrasound provides a high cleaning performance of the UV-tubes.
- As ultrasound effects are limited to the treatment plant components, cavitation effects on the ships' piping and tank system are not possible, whereas the treatment plant components are designed to cope with cavitation.
- By the application of UV-disinfection micro-organisms cannot develop resistance; therefore also resistant pathogens (e.g. chlorine resistant) are inactivated by UV-C radiation.
- UV-C radiation and ultrasound do not effect physical parameters like salinity or pH-values of the ballast water.

2.3 Persons in charge of the project at NIOZ

Name	*Position at NIOZ*	*Project duty*
Dr. Marcel Veldhuis	*Senior scientist, Department of Biological Oceanography*	*Co-ordination of scientific program and biological tests under Guideline G8*
Dipl.-Biol. Frank Fuhr	*PhD student*	*Sample analysis and scientific evaluation of semi-automated sample analysis*
Peter Paul Stehouwer MsC	*PhD student*	*Sample analysis and scientific evaluation of semi-automated sample analysis*
Isabel van der Star	*PhD student*	*Sample analysis and scientific evaluation of semi-automated sample analysis*

For biological studies:

Eveline Garritsen, marine chemistry

Anna Noordeloos, marine biologist, (phytoplankton ecology)

Swier Oosterhuis, marine biologist, (general plankton ecology)

Josje Snoek, marine biology/chemistry

For specific laboratory assistance for DOC, nutrients, salinity, POC, Oxygen:

Jan van Ooyen (senior technician)

Karel Bakker

Evalien van Weerlee

Santiago Gonzalez

2.4 Land based test site

The land-based tests will be carried out on the island of Texel (NIOZ harbour, NL) from April to July 2009. The NIOZ test-site is equipped with 2 coated silos of 300 m^3 each to simulate the ballast water tanks of the ship. Slightly modified natural water is going to be used during the tests (see above). At the NIOZ test site this water is originating from the Wadden Sea (North Sea). The numbers of organisms present in the water at the test site is to be monitored before and during the tests in order to assure the validity of the test cycles in accordance with 2.3.19 of G8.

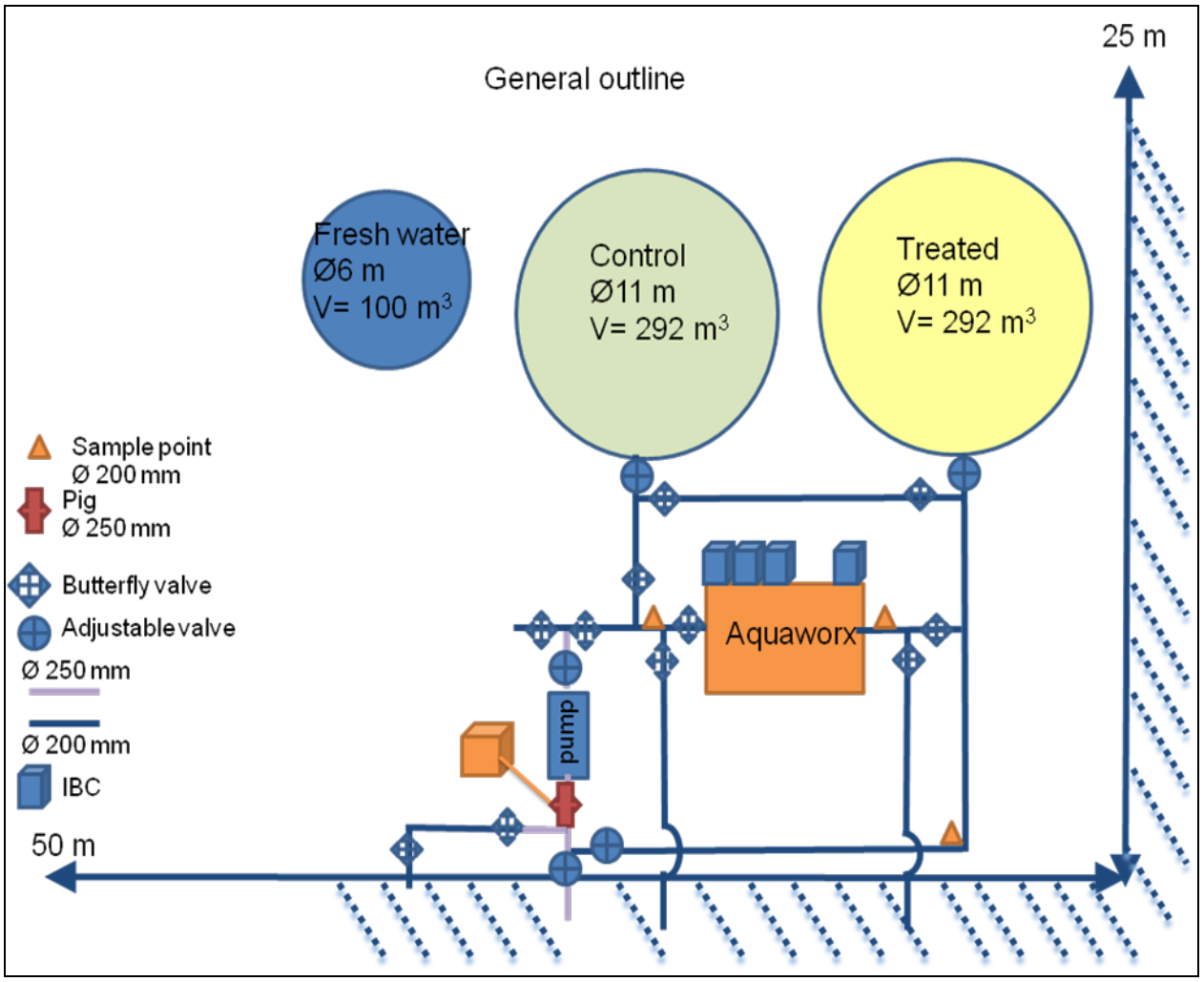

Figure 3 P&I of the Test site at the NIOZ harbour, Texel Island, The Netherlands.

3 TEST PROTOCOLS

The **land based tests** have to be carried out at specific water qualities. The NIOZ-harbour represents a brackish water environment (3 – 32 PSU).In order to cover the requested two out of three salinity ranges of the table under Paragraph 2.3.16 of G8.[1] Salt will be added to the natural water to increase the salinity above 32 PSU. Since it is also requested that tested salinities must be at least 10 PSU apart, freshwater may be added to lower salinities for the second series below 22 PSU. A valid test cycle is characterized by the following challenge water conditions:

3.1 Challenge water quality

An overview of the water quality, with respect to the composition of total suspended solids, particulate organic carbon and dissolved organic carbon is given in Table 1.

Table 1: salinity ranges and minimum concentrations of TSS, POC and DOC in the challenge water.

	Salinity		
Parameter	**> 32 PSU**	**3 – 32 PSU**	**unit**
Total Suspended Solids	**> 1**	**> 50**	**mg/L**
Particulate Organic Carbon	**> 1**	**> 5**	**mg/L**
Dissolved Organic Carbon	**> 1**	**> 5**	**mg/L**

3.2 Numbers of organisms

In order to test the efficiency of the Aquaworx™ BWT-system it should be tested with water containing a high density of organisms as well as sufficiently great diversity (2.3.16 of G8). This is required to guarantee the effectiveness of the BWT-system across the globe and in a variety of environmental conditions. The variety of organisms in the influent test water should be documented according to the size classes mentioned below (Table 2).

Since natural water, originating from the coastal zone of the North Sea and the inner Western Wadden Sea will be used with the ambient plankton content the number of species will be very high, usually over 50 species. In addition the test period will cover the whole spring and early plankton growth season and therefore includes the natural occurring biodiversity and species succession.

Table 2: Minimal numbers and species diversity required at intake for different size classes and groups of organisms.

	Influent test water	
Parameter	**unit**	**Remarks**
organisms ≥ 50 micron	**> 10^5/ m^3**	**at least 5 species from at least 3 different phyla/divisions**
10 ≤ organism size ≤ 50 micron	**> 10^3/ mL**	**at least 5 species from at least 3 different phyla/divisions**
heterotrophic bacteria	**> 10^4/ mL**	**not further defined**

For completeness, the planktonic fraction < 10 µm is also included in the tests.

[1] References as indicated in Annex 3 – Resolution MEPC.125(53) G8; Annex Part 1,2,3, and 4)

The natural waters of the test area include a large range of organisms varying in sensitivity to mechanical and UVC radiation. Besides fragile organisms there will be also organisms highly adapted to harsh environmental conditions (most hard shell species).

3.3 Quality management (QA/QM)

In principle NIOZ is a scientific research institute participating in numerous international projects whereas measurements according to high approved standards are required.

Moreover, samplings for long-term series of physical, chemical and biological data also require a high standard of QA/QC. During this project all samples are taken, stored and analysed according to **international defined standards** whenever applicable.

For specific equipment, e.g. flow cytometers (a medical instrument meeting FDA standards), the performance of the instrument, like machine drift and flow rate using **ISO certified standards** (10.03 ± 0.03 μm and 49.7 ± 0.7 μm polymer microspheres, Duke Scientific corporation and Flow Check Fluorospheres Beckman Coulter) is checked according to the manufacturers recommendation.

Samples for the human pathogens are taken with great care to avoid contamination and shipped in special bottles provided by the test laboratory (Eurofin/C-mark) immediately after sampling to the test laboratory.

For numerous parameters, mainly concerning the biological components, **multiple methods** are applied for counting and determining the viability of the organisms. This includes text book methods as well as some promising state of the art technologies for application in BWT research. In the upcoming period results of these different methods will be compared in great detail and these results will be made public by means of publications in international peer reviewed papers and/or submitted to official authorities for evaluation. For the time being present results are based on the most conservative of the methods applied.

Since the test site is within short distance of the research facility all samples containing fresh and life material are immediately transported to the laboratory in special cool containers for direct analysis.

Each of the participating scientists and technicians, with exception of the master-student and the Ph-D student, is an expert in its specific field for at least 10 years.

4 MEASUREMENTS

4.1 Basic parameters / water quality

The monitoring of the following basic parameters is required during **land based testing**: Salinity, temperature, Particulate Organic Carbon (POC), total suspended solids (TSS), pH, dissolved oxygen and Dissolved Organic Carbon (DOC).

4.2 Salinity

For salinity ca. 250-500 ml of water is sampled and stored at room temperature (glass bottles) until analysis through either direct measurement in the laboratory (at NIOZ).

4.3 Temperature

Will be measured using a calibrated thermometer (electronic or ordinary type).

4.4 pH

Will be measured using a calibrated pH meter.

4.5 Turbidity

Turbidity is measured in **N**ominal **T**urbidity **U**nits (NTU) according to EN ISO 7027.
Turbidity is the reduction of a liquid caused by the presence of undissolved matter. Measurements of turbidity can be performed at wavelengths greater than 800 nm. The EN ISO 7027 specifies two quantitative methods for the determination of turbidity in water, using optical turbidity meters:

- Measurement of a diffuse radiation
- Measurement of the attenuation of a radiant flux. The latter measurement will be applied at a wavelength of the incident radiation of 860 nm.

4.6 Dissolved Oxygen (DO)

Gastight tubing which is specially fitted to the sampling tubing is used to avoid exchange of gasses between water and surrounding atmosphere (figure 4).

Figure 4: Sampling point at tank 3 for sampling gasses and volatile substances

The coded glass bottles are flushed at least three times their volume (ca. 120 ml) with water. 1 mL of solution I ($MnCl_2$) and 2 mL of solution II (NaOH) are added to the sample below the surface by using dispenser units. The dispenser units are wiped clean after each sample to avoid any contaminations.

A stopper is put on the bottle and the bottle is gently mixed.

The stopper is secured with a rubber band and then the bottles are stored in a dark container filled with water of the same temperature as the samples until further analysis (figure 5).

Figure 5: glass bottles for DO measurements stored submerged prior to analysis.

Prior to the actual measurement 1 mL sulfuric acid (H_2SO_4) is added and the precipitation is dissolved again in a chemical reaction which releases iodine. The absorption of the fluid is measured at 456 nm. Oxygen concentration is calculated using a calibration curve. Oxygen concentration is expressed as mg O_2 per L.

4.7 Active substance

For the reason that the AquaTriComb™ treatment system is <u>not</u> generating active substances in the sense of chemical substances (photons and cavitation bubbles as physical relevant procedures) Other Chemicals per definition of G8 and G9 are to be considered. Aquaworx ATC GmbH has done detailed analytics regarding possible by products within the preparation of the Basic Approval. Results of chemical analysis of treated water samples showed for 32 substances and substance groups, that for none substances elevated concentrations compared with background levels in the harbor water could be detected.

Hence during the treatment process none chemicals are neither added nor generated by the AquaTriComb™ treatment system.

In general UV-rqdiation is producing no active or harmful by- or end products. Previous studies however showed that under certain conditions the concentration of NO_2 can be elevated. For that reason the main nitrogen sources will be determined (NO_3, NO_2, NH_3).

4.8 TSS/POC (total suspended solids and POC)

For TSS/POC pre-weighted filter (GF/C) are used. Each filter is coded and separately stored in a Petri dish. The filtered volume is dependent on the concentration of particles of the sample. The higher the particle load is in the sample, the smaller is the volume that can be filtered before the filter is clogged. Practical volumes are between 200 and 1500 ml per sample. After filtration the filter is rinsed with **fresh** water to remove sea salt. The filter is then put back in its original Petri dish and stored in a freezer at –20°C until further analysis (POC is analysed by NIOZ).

For TSS the filters are dried at 60°C for 24 hours and then weighed again. The concentration of TSS per litre can then easily be calculated from the sample volume and the weight difference of the filter before and after. TSS is expressed as mg per L.

For POC the filter is combusted at 500°C (overnight) and allowed to cool in a vacuum exicator and weighted again. The POC is calculated from the weight decrease between this measurement and the TSS weight. POC is expressed as mg C per L.

4.9 Dissolved Organic Carbon (DOC)

The concentration of dissolved organic carbon (DOC) will be measured according to Reintaler & Herndl (Reinthaler & Herndl 2005). Samples for DOC (20 mL) are filtered through GF/C filters and sealed in pre-combusted glass ampoules after adding 50 µl of phosphoric acid (85% H_3PO_4). Sealed ampoules are stored at 4°C. The DOC concentration was determined in the laboratory by the high temperature combustion method using a Shimadzu TOC-Vcpn analyzer. Standards were prepared with potassium hydrogen phthalate (Nacalao Tesque, Inc, Kioto, Japan). The mean concentration of triplicate injections of each sample (three in total) is calculated. The average analytical precision of the instrument is < 3 %.

4.10 Biological analysis during land based tests

As indicated above the present ballast water treatment system differs from other systems in the fact that treatment of the ballast water is not only during intake but also at discharge. Inspection will be immediately at intake (T0), at discharge (T5) and 5 days after discharge (T10).

In addition to sampling large scale basins for a period of 5 days subsamples (volume of 10 L) of the reference and treated tank will be taken at intake and discharge and incubated in a climate room under optimal growth conditions for the plankton community as present in the test water. In case the treatment is insufficient and residual viable organisms remain present, or there is germination of resting stages or cysts, growth of the plankton will be stimulated under these favourable conditions. This incubation method also allows studies on the effect of the treatment over a period longer than 5 days (up to 20 days). A sufficiently high level of nutrients will be maintained, favouring phytoplankton growth and possible cyst germination throughout the incubation experiments.

The majority of the large size fraction (>50 µm) consists of zooplankton, while the majority of the small size (10-50 µm) fraction consists of phytoplankton. Organisms > 50 µm are retained as recommended in MEPC 54/Inf.3 (using a Hydrobios net).

Samples for the 10 µm < organisms <50 µm fraction are coming from the effluent of the Hydrobios net. This size-fraction is not separated from the organisms < 10 µm.

For both of the IMO relevant size classes (organisms >50 µm and organisms 10 – 50 µm), multiple methods of enumeration and of assessing the viability are applied to verify the results at a high level of confidence. In principle all methods should give a conclusive answer with respect to numbers and/or viability of the (remaining) organism.

4.11 Sample sizes

During the **land-based tests**, the following sample sizes will used:

Control: 1 container of 500 L, allowing for sample volumes of 3 times 20 L + 3 times 1 L and 3 times 1 L .

Treated (intake): 3 IBCs (>1000 L), allowing samples of 1 m^3 each, + 3 times 10 L and 3 times 1 L.

Treated (discharge): (after 5 days of retention time): 3 IBCs (>1000 L), allowing samples of 1 m^3 each, + 3 times 10 L and 3 times 1 L.

The above sample sizes assure availability of sample volume as outlined in Paragraphs 2.3.30 through 32 of G8 for the different size classes of organisms:

≥ 50 µm:	20 L for untreated water and 1000 L for treated water.
<10 and 10 – 50 µm size range:	1 L for untreated water and 10 L for treated water.
Bacteria:	1000 mL.

4.12 Organisms > 50 µm

Organisms in this size class are analyzed using two methods. Firstly standard manual counting of organisms and secondly, as means of quality insurance, a semi-automated method (FlowCam) is applied.

Note: for practical reasons all organisms retained on the sampling 50 µm sampling net are considered as larger than 50 µm in minimum dimension.

4.13 Collection of samples for manual counting

The samples are collected using Hydrobios 50 µm nets which are fitted into 500 L containers (see figure 6). Sampling is conducted via flexible hoses which are connected to the sampling points. For sampling of the treated water, the hose is put into the Hydrobios 50 µm net. The 500 L containers are filled twice with each individual sample, resulting in a sampling volume of 1000 L for each of the samples. The whole sampling procedure will take ca. 1 h, and therefore covers the whole period of filling the ballast water tank with ca. 200 tons of water.

The samples of the untreated water (20 L) are taken in triplicate and collected in clean buckets that are filled directly for volumetric measurements and are then poured through the net.

Figure 6: Sampling containers of different sizes at the NIOZ harbour (500 L and 1 m^3). A 50 µm Hydrobios net is set up in the containers using a modified lid for easy sampling.

4.14 Processing of samples for manual counting

The organisms retained in the cod-end of the Hydrobios net are flushed into a heat sterilized beaker using a squeeze bottle (containing filtered seawater). Organisms are kept in approximately 100 to 200 ml of filtered (= organism free) water of the relevant salinity. The samples are transferred to the lab directly after sampling and Neutral Red is added (Eppendorf pipette) in a ratio that yields an end concentration of approx. 1:50.000 (Stock solution: 1:2.000, i.e. approx. 2 ml of stock solution are needed to stain 50 ml of water). Staining time is 2+ hours. Neutral Red stains **living** organisms only and does not affect the survival rate. Therefore the viability assessment remains unaffected by the possible death of organisms during the staining or during sample analysis due to warming of the sample etc. This is due to organisms dying after addition of the Neutral Red will be clearly stained, while those already dead prior to this will not.

Neutral Red does work reliable for all major groups (this includes phytoplankton as far as it was encountered up to the present date), but it seems to have some limitations for bivalves. For the latter in addition movement (including internal like heart and gills for juvenile mussels) can be used to verify viability. This is dependent on the expertise of the person analysing the samples. Therefore, wherever possible, the same person should analyse all samples of one series.

The stained samples are filtered over a 30 µm sieve and flushed into a Bogorov-like with filtered water. The samples are analysed using a binocular with a 20x magnification for counting and up to 50x for species identification and measurements when necessary.

Organisms need to be counted according to their size. Here organisms of 50 micron in minimum dimension are relevant. Several tests have shown that a single size bar is not efficient as viable organisms move in the counting chamber. Better results are achieved when the entire field of view is equipped with a size grid. Earlier tests showed that a practical approach is to place a piece of the 50 micron net below the counting chamber. The net is transparent, i.e. no organisms are overlooked when they lay directly on the mesh lines. By doing so the entire field of view shows a 50 micron grid and allows counting of organisms above 50 micron only.

For minimum dimension measurements the "body" of the organism should be measured, i.e. not antennae, tails etc. Examples are presented in figure 7.

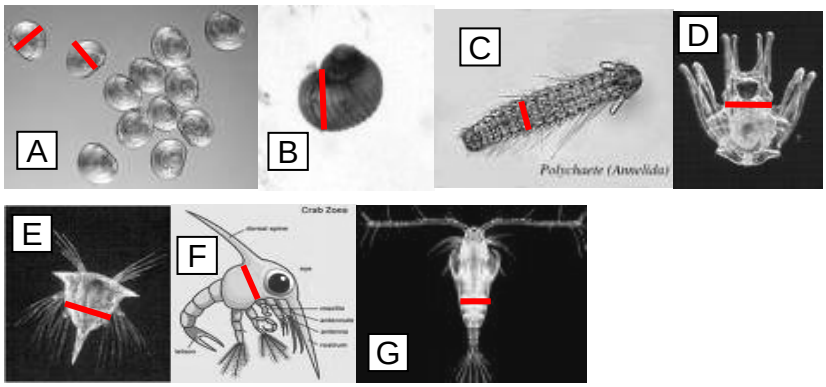

Figure 7: Minimum dimension measurements (red line) in selected organism types:
A = bivalve larvae, B = gastropod larvae, C = worm, D = echinodermata larvae, E and F = crustacean larvae and G = copepod.

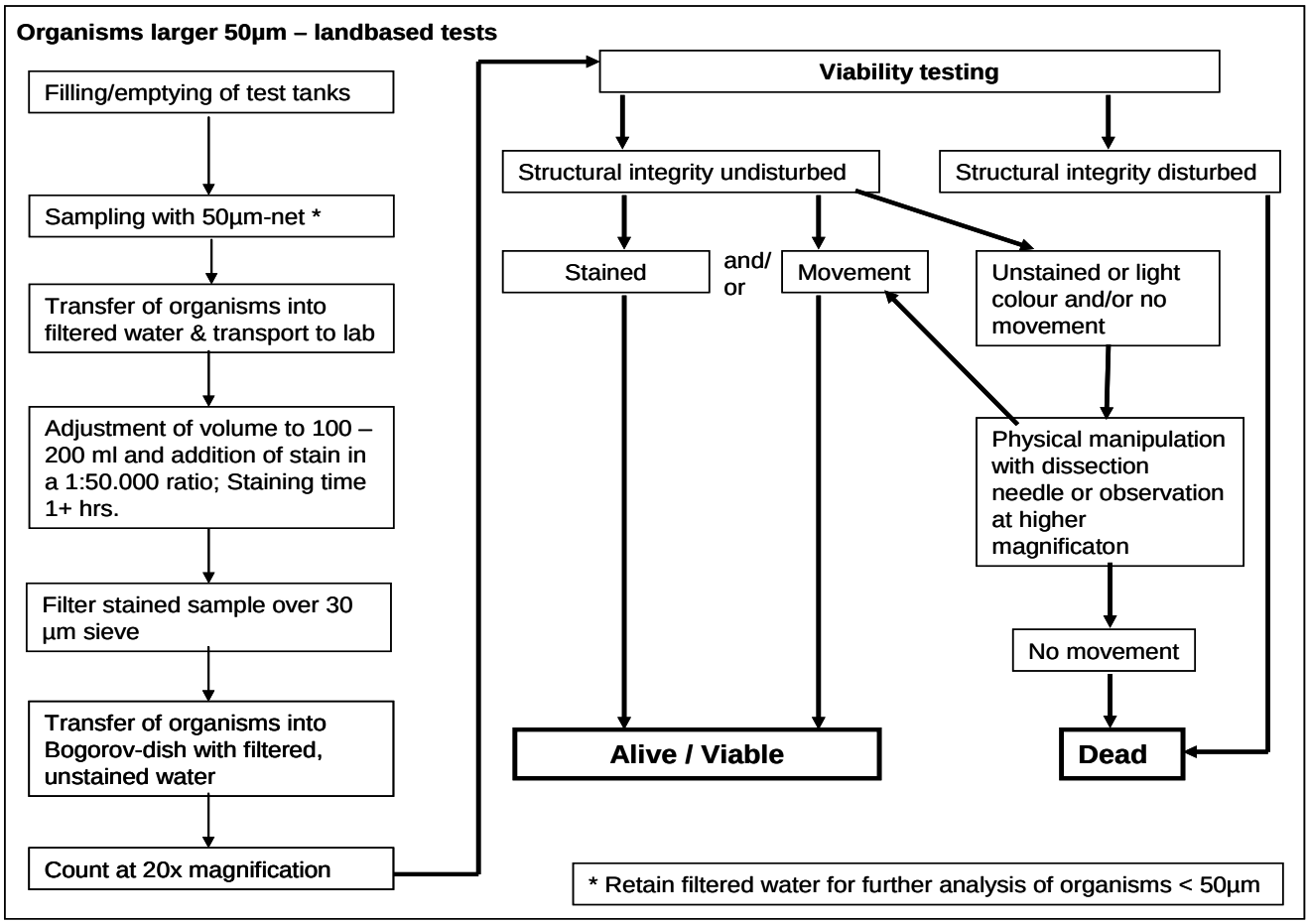

Figure 8: Sampling and viability assessment for organisms larger than 50 µm during land-based tests

4.15 FlowCam

The FlowCam used in the tests is an improved model of the standard FlowCam (Fluid Imaging Technologies, Edgecomb, ME, USA, figure 9). The modifications are specially made for testing ballast water and include the following adaptations; 1) enhanced sensitivity by using a blue laser (473 nm solid state laser) for phytoplankton detection, 2) a high sensitive colour camera for improved pictures of morphological features and colour, 3) detailed colour analysis of organisms after staining with the viability specific dye Neutral Red (applied in a concentration of 1:50.000, identical to the microscopic method).

Figure 9: portable version of the Flow Cam, picture Fluid Imaging Technologies

Whole samples will be taken, 1 L for control and 10 L for treated water and concentrated using the Hydrobios sampling net designed for ballast water testing. Samples will be stored in a cool box in the dark and measured almost immediately at the laboratory. Of each subsample the whole intact sample will be analyzed (qualitatively) by the FlowCam using the automated mode. Since this method is non-destructive the same sample will be analyzed also microscopically. Of all particles (exceeding an average cell diameter of 5 µm) a full colour picture will be taken and stored at the hard disk, for later inspection by means of the morphological features.

Because of its design FlowCam is not capable to count all particles present. For this reason samples will be analyzed several times to check for selective counting errors. Samples are also counted manually and these results will be compared with the microscopic determination of species composition, cell number and particle integrity. In this manner the general counting efficiency of the instrument can be determined. Moreover, non-toxic spherical beads with different sizes will be added to the sample in a known quantity as an internal standard. Because of their specific nature these beads can easily be detected using the inbuilt image analysis program of FlowCam. Thus counted numbers will be compared with the amount of beads added to determine the counting efficiency.

4.16 Organisms > 10 µm and < 50 µm and < 10 µm

Organisms in this size class will be analyzed via inverted microscopy and flow cytometry (figure 10). The latter is a semi-automated method used for counting of phytoplankton, bacteria and viruses. Additionally the phytoplankton component is further characterized by PAM fluorometry and measurement of the plant pigments and chlorophyll *a* content.

Figure 10: Bench top Flow cytometer; a fast and reliable method to enumerate and determine the size of planktonic organisms.

4.17 Collecting of samples for inverted microscopy

The samples for organisms smaller than 50 µm in the smallest dimension are taken from the effluent (i.e. passing the 50 µm Hydrobios net) of the above samples. According to the requirements, the following sample volumes are collected: 1 L in triplicate from untreated water (e.g. control and influent water) and 10 L in triplicate from the treated water.

4.18 Processing of samples for inverted microscopy

The samples are filtered using a sieve with a Hydrobios 10 µm gaze. Effective size range is 10 – 50 µm. The retained organisms are flushed into 50 ml Greiner tubes using 30 ml of filtered water and fixed with Lugols solution for later inspection with the inverted microscope. These samples can be stored in the dark at room temperature for up to one year.

The Samples are analyzed with an inverted microscope at 200x magnification (method by Utermöhl). The fixed samples are transferred into settling chambers and left undisturbed in the dark for at least 8 hours before counting. The iodide component of the Lugols solution that stains organic particles in the sample also increases their weight. Therefore they settle on the bottom-glass and can be counted. Live-dead-separation in these samples is mainly based on the structural integrity of organisms. This method can be applied for both zoo- and phytoplankton. Nevertheless certain groups like ciliates are known to be severely affected by this standard method of fixation and therefore will be systematically underestimated, if present. The sampling and processing of the samples is summarized in figure 11.

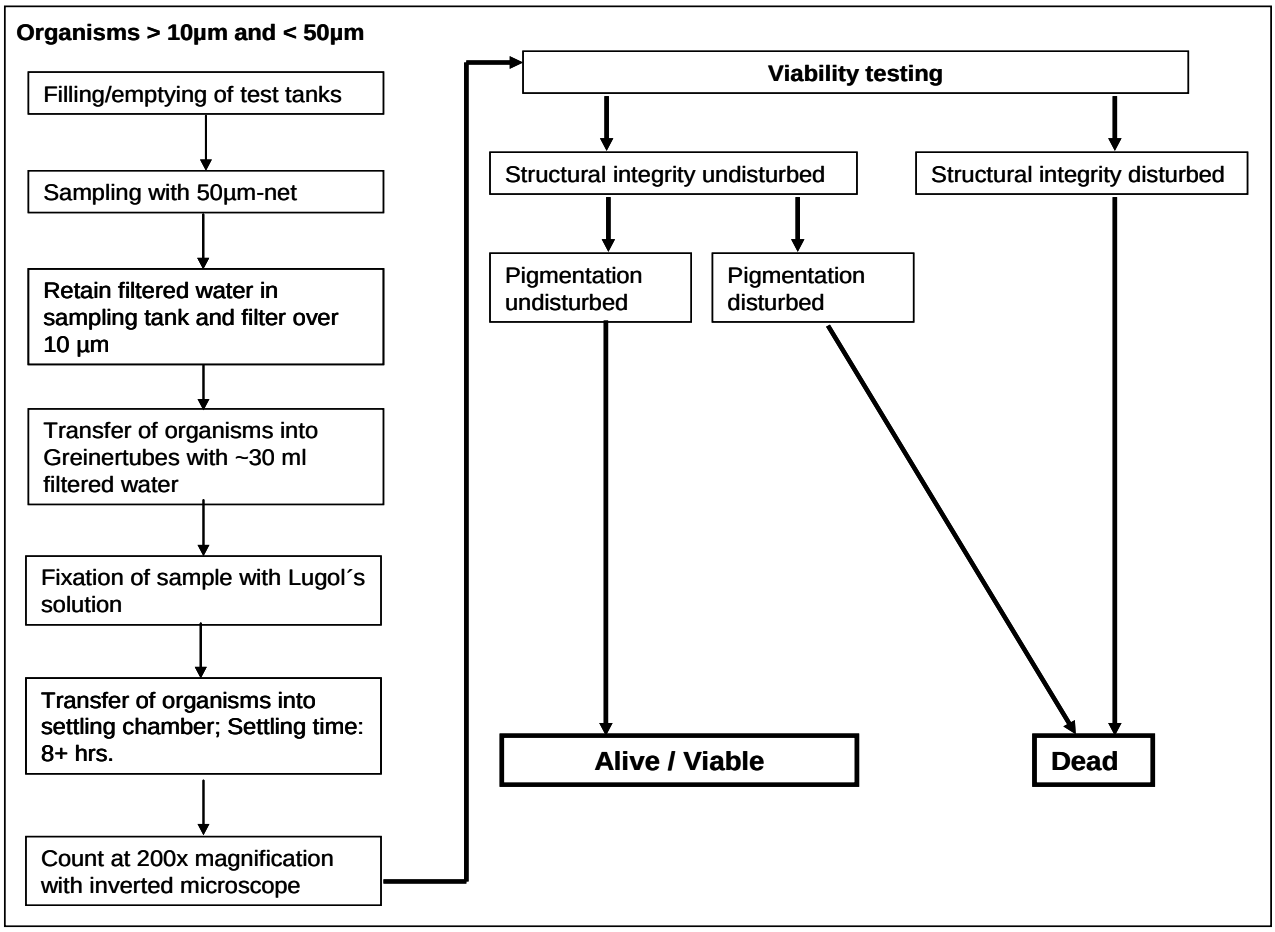

Figure 11: Sampling scheme and viability assessment for organisms smaller than 50 µm

4.19 Semi-automated methods

The size fraction of organisms between 10 µm and 50 µm in the smallest dimension is analyzed by flow cytometry, as a quantitative indicator of plankton number and size. Because of its design and differences in numerical abundance flow cytometry is in particular useful for phytoplankton and will also include the organisms <10 µm. This will allow us to have a complete view of effectiveness of the applied treatment technique for all organisms, irrespective of their size. By applying special staining techniques also the numerical abundance of the bacterial community will be estimated. The size-specific viability of the different organisms present will be addressed using the method described by Veldhuis et al. (Veldhuis et al. 2001; Veldhuis et al. 2006).

4.20 Flow cytometry:

Whole, intact samples (1 L for control and 10 L for treated water) will be taken fresh and stored in the dark prior to analysis in the laboratory.

Three replicate samples of 3 mL each will be taken from both control and treated water. They will be pipetted in an ultra clean sampling tube and put in the carousel of a bench top flow cytometer (Beckman Coulter XL-MCL). This flow cytometer is certified by the FDA for medical laboratory use and as such maintained by the manufacturer in their service scheme. All procedure and handling are conducted according the standard procedures as described in Shapiro (Shapiro 2003). As a light source a 15 mW Argon laser is used (488 nm excitation wavelength). Forward and side scatter is detected of each particle as well as the fluorescent emission in the yellow/green (525 ± 20 BP filter), orange (575 ± 20 nm) and red wavelength band (>645 nm, for details see Veldhuis & Kraay 2000). Samples will be counted using a standard protocol covering the particles in the size range of ca. 2 to 50 µm. Total analysis time will be equal to an exact sampling volume of 1 ml (see volume calibration for details). Of all particles present in 1 ml of sample, cell size and presence or absence of chlorophyll a will be measured. Absolute numbers, cell sizes and chlorophyll *a* content of the particles will be analyzed using the software package FCS Express V3 (DeNovo version 3). Cell sizes will be estimated relative to 10 µm standard fluorescent beads (Flow-Check Fluorospheres, Beckman Coulter #660539). Phytoplankton will be distinguished from other

particles based on the presence of chlorophyll *a*, resulting a bright red fluorescent signal (emission > 645 nm).

The flow cytometer will be calibrated volumetrically and with an internal standard. The flow rate is determined by weight of the samples prior and after analysis. The sensitivity of the instrument will be determined using standard calibration beads (Flow-Check Fluorospheres, Beckman Coulter #660539). These beads have an exact size and fluorescent signal with a CV for the different parameters of < 2% (HPCV). These standards will be run every sampling day, however over 14 years of experience has shown that instrument stability is extremely high over periods of months. Since the samples of interest are loaded with a high content of detrital material total and group specific number of organisms will be corrected for blank counts. These are so called UFO's (unidentified fluorescent objects) using a artificial made soup of detritus using 50 mg/l of organism free suspended matter. Since the addition of dyes like SYTOX Green increase the general fluorescence signal of all particles this calibration will be done both with and without the dye.

For the viability testing, subsamples (3 in total) of 1 ml will be taken and pipetted in ultra clean sampling tubes. To this sample volume 10 µl of (100 fold stock dilution of SYTOX Green) will be added (Veldhuis et al, 2001, Casotti et al, 2005). This nucleic acid specific dye only stains DNA of intact cells with a compromised cell membrane (figure 12). Sample will be incubated for 10 to 15 min prior to analysis. Of each phytoplankton present in the untreated sample the yellow/green fluorescence will be determined and compared with the yellow/green autofluorescent signal (cf. Veldhuis et al, 2001). A phytoplankton cell is classified as non-viable if the yellow/green fluorescent signal is at least 5 times higher than the autofluorescent signal of the cell. Cells will be classified in separate clusters, when present according to their size distribution and numbers of viable and non-viable cells will be correct for blank no-specific staining.

Figure 12: (A) Epifluorescence microscopic picture of a live phytoplankton cell. The red signal is due to the presence of chlorophyll and (B) a dead phytoplankton cell with a yellow/green fluorescence of the nucleus after staining with SYTOX Green.

4.21 PAM fluorometry:

The photochemical efficiency of photosystem II (an indicator of the 'health' condition of the cell) of phytoplankton can be addressed using a Pulse-Amplitude Modulated fluorometer (PAM-fluorometry; Schreiber et al 1993, figure 13). This simple parameter gives a qualitative indication of the photosynthetic activity of the phytoplankton community. For this 3 ml of unfiltered sample water (control and treated, each in triplicate) are filled into a glass cuvette and analysed using the Pulse-Amplitude Modulated fluorometer (automated measurement). In case an increase of the photosynthetic activity of the whole phytoplankton community is observed samples will be filtered (50 and 10 µm Hydrobios sieves) to determine the exact size class of the viable phytoplankton fraction.

Figure 13 PAM fluorometry; a fast method to determine (bulk) phytoplankton biomass and the physiological condition of the photosynthetic apparatus of the cells.

4.22 Plant-pigments and chlorophyll *a*

Separate water samples of 0.2 to 1.5 l (GF/C filters) are taken for measurements of **total plant pigments** and **chlorophyll *a*** as an indicator for total phytoplankton abundance and species composition using High Pressure Liquid Chromatography (HPLC, cf Jeffrey et al. 1997; Kraay et al. 1992, figure 14). The samples are frozen until further analysis (-80°C). The system used is a Dionex HPLC system equipped with a C18 separation column. The different algal pigments can be separated according to their polarity. The following solvents are used as elutes in the HPLC gradient: A 0.5M ammoniumacetate in methanol and water (85:15), B acetonitril and water (90:10) and C ethylacetate 100%.

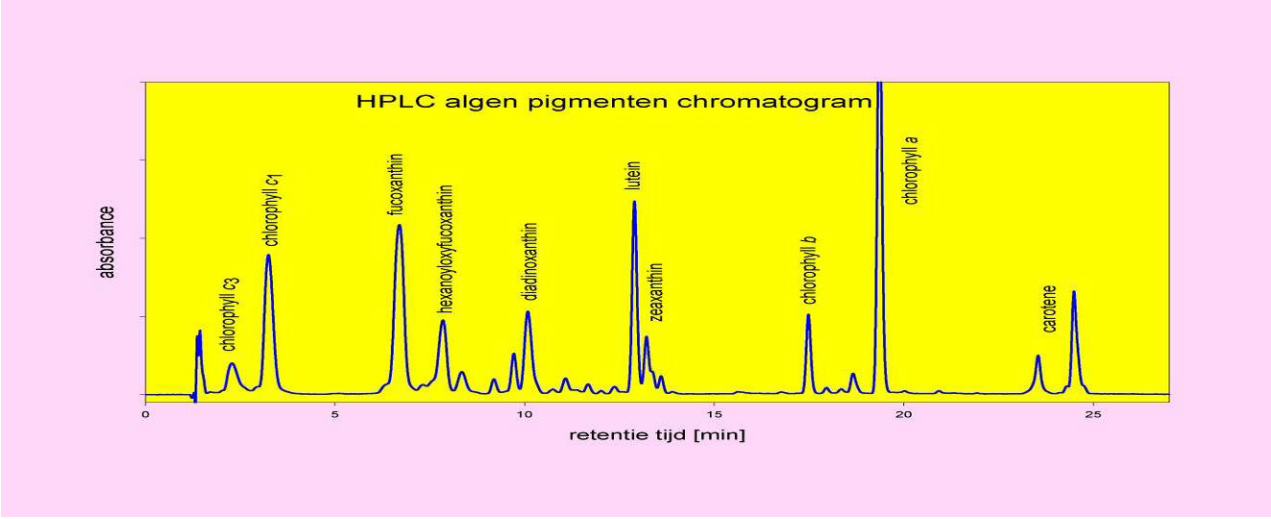

Figure 14 HPLC chromatogram showing a typical composition of phytoplankton main and accessory plant-pigments.

To the filter containing the plant pigments an extraction solution of methanol containing ammoniumacetate (2%) is added. Together with glass pearls (appr. 350 µm spherical diameter) this mixture is vigorously shaken under CO_2 cooling, using a Braun homogenizer. The homogenate containing the extracted pigments is injected in the system after which the resulting chromatogram is analysed. On average 20 different pigments can be recognized. The results of the analysis are used in a statistical

classification program, CHEMTAX (Mackey et al. 1996), that enables the recognition of the different phytoplankton class abundances.

4.23 Total bacteria

The classical method for counting bacteria in many applications is based on plating on selective media. Unfortunately, for studies in the aquatic environment this approach is by far insufficient for various reasons (Gasol & Giorgio 2000). As a result total bacteria will be determined by flow cytometry, using DNA-specific stains to get a more accurate bacteria number. In addition samples were taken at discharge for specific human pathogens and heterotrophic bacteria using a plate method.

To determine the total bacteria number a 1.5 mL water sample is taken and pipetted in a Cryovial (in triplicate) and formaldehyde (100 µl of a 36% solution) was added as a preservative. Samples were frozen and stored at -80 °C until further analysis.

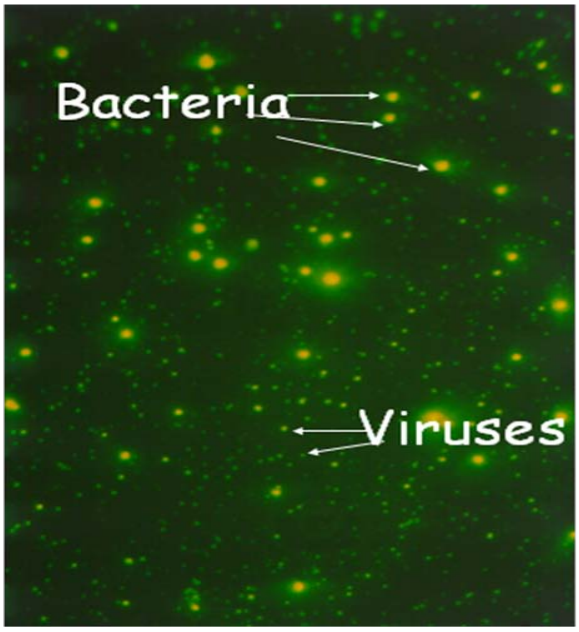

Figure 15: epifluorescence microscopic picture showing bacteria and viruses in a natural water sample. yellow-green fluorescence signal is of the DNA stained with a nucleic acid dye.

Upon analysis the sample is allowed to thaw completely. A subsample of 100 µL is taken, diluted with a TE-buffer, and the nucleic acid dye PicoGreen (Invitrogen) was added. Within 5 to 15 minutes after the addition of the stain the sample is analyzed using a flow cytometer (cf. Gasol & Giorgio 2000; Veldhuis et al. 1997). A know bacterial standard is used for calibration and counting.

The number of heterotrophic bacteria will be determined using a plate method as the number of colony forming units (cfu's) after incubation of the water at intake and discharge according to an international standard (NEN-EN-ISO 6222:1999).

The dye PicoGreen is a green nucleic acid specific dye that only stains dsDNA, with little or no cross-over for ssDNA and RNA (Veldhuis et al, 1997). This makes the staining method ideal to for staining of DNA and therefore to determine bacterial abundance. Flow cytometric analysis shows a clear signal with an excellent signal to noise ratio and bacteria are made visible easily and distinguishable from viruses and larger organisms. This approach has extensively been compared with bacteria staining and counting using an epifluorescent microscope, resulting in nearly identical results. However, since the flow cytometer method is much faster (results are obtained within 100 seconds), highly reproducible and over 100 samples can be analyzed per day this bacteria counting method is to be preferred above the far more time consuming microscopic observations.

4.24 Human pathogens

The samples for microbiological analysis are taken in special bottles of 300 or 600 mL and send to a special laboratory (eurofins/ C.mark) for further analysis. The final measurement are conducted by "Vitens laboratory bv" at Leeuwarden (accreditation certificate: NEN/ISO\IEC 17025; lab. no. L043). All analysis' are carried out according to NEN/ISO standards.

4.24.1 Escherichia coli:

Analysis for *Escherichia coli* is carried out according **to ISO 9308-3** for the analysis of surface waters. For this the samples are filtered through membrane filters (pore size 0.45 µm) and these filters are incubated on a selective agar plate. Incubation is 4,5 ± 0,5 hours at 37 ± 1°C and then another 19,5 ± 0,5 hours at 44 ± 0,5 °C. After that the incubated filters are transferred on sterile filters soaked with Indol reagent. For colonies of *E. coli* this yields a red colour. These red colonies are counted and set into relation to the sample volume. Results are confirmed via a positive and a negative control. For the latter sterilized water is incubated like a regular sample and to confirm the results it may only yield less than 1 colony forming unit (cfu) per 100 ml. The positive control uses a special strain of *E. coli* also incubated like a normal sample to confirm that this species can grow and form colonies on the used media.

4.24.2 Enterococcus group:

Analysis for this group is carried out according **NEN/ISO 7899-2**. For this the samples are filtered through membrane filters (pore size 0.45 µm) and these filters are incubated on a selective agar plate. Incubation is 44 ± 4 hours at 36 ± 2°C on Slanetz & Bartley medium. After that red and pink colonies are counted. If the presence of enterococcus bacteria can be suspected after the colour of the colonies the filter is transferred to a pre-heated, selective agar plate and incubated for another 2 hours at 44 ± 0,5 °C. After that the medium is examined whether or not a brown to black colour can be found in it. Results are confirmed via a positive and a negative control. For the latter sterilized water is incubated like a regular sample and to confirm the results it may only yield less than 1 colony forming unit (cfu) per 100 ml. The positive control uses a strain of *Enterococcus faecium*.

4.24.3 Heterotrophic bacteria:

Analysis for this group is carried out according to **NEN-EN-ISO 6222 (2nd imprint 1999**). For this the samples are pipetted into Petri dishes and mixed with the soft melted medium (yeast extract agar at 45 ± 1°C). Two different incubation strategies are used for each sample. One is incubated for 44 ± 4 hours at 36 ± 2°C, while the other is incubated for 68 ± 4 hours at 22 ± 2°C. After that the present cfu's are counted. Results are confirmed via a positive and a negative control. For the latter sterilized water is incubated like a regular sample and to confirm the results it may only yield less than 1 colony forming unit (cfu) per 100 ml. The positive control uses strains of *Pseudomonas fluorescens* and *Enterococcus faecium*.

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